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ATTN: MCMR-CDC-C
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

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ATTN: MCMR-CDZ-I
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Glutamate receptor pathology is present in the hippocampus following repeated sub-lethal soman exposure in the absence of spatial memory deficits

Erik A. Johnson [a,b,*], Kelly S. Daugherty [b], Sarah J. Gallagher [b], Anita V. Moran [b], S. Michelle DeFord [b,c]

[a] US Army Medical Research Institute of Chemical Defense (USAMRICD), 3100 Ricketts Point Road, Comparative Medicine Division, Comparative Pathology Branch, Aberdeen Proving Ground, MD 21010-5400, USA
[b] US Army Medical Research Institute of Chemical Defense (USAMRICD), Analytical Toxicology Division, Neurobehavioral Toxicology Branch, Aberdeen Proving Ground, MD 21010-5400, USA
[c] Department of Psychology, University of Massachusetts at Dartmouth, 285 Old Westport Road, North Dartmouth, MA 02747, USA

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Abstract

Much is still unknown about the long-term effects of repeated, sub-lethal exposure to organophosphorus (OP) nerve agents, such as soman (GD), on learning and memory tasks and related protein expression in the hippocampus. In the present study, guinea pigs were exposed to sub-lethal doses of GD for 10 days and cognitive performance assessed using the Morris water maze (MWM) up to 88 days post-exposure to investigate spatial learning. Additionally, hippocampal lysates were probed for cytoskeletal, synaptic and glutamate receptor proteins using Western blot analyses. No significant difference in MWM performance was observed between repeated sub-lethal GD exposed and saline control groups. However, Western blot analyses revealed significant changes in glutamate receptor protein immunoreactivity for subunits GluR2, NMDAR1, NMDAR2a and NMDAR2b in the hippocampi of GD-exposed guinea pigs. Levels of GluR2, NMDAR2a and NMDAR2b increased by 3 months post-initial exposure and returned to control levels by 6 months while NMDAR1 decreased by 6 months. No significant differences in neurofilament medium (NFM), neurofilament light (NFL) or synaptophysin densitometry were detected and α-II-spectrin proteolytic breakdown was also absent. These results reveal that repeated, sub-lethal exposure to GD affects glutamate receptor subunit expression but does not affect cytoskeletal protein immunoreactivity or the proteolytic state in the hippocampus. Though these changes do not affect spatial memory, they may contribute to other cognitive deficits previously observed following sub-lethal OP exposure.
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Keywords: Soman; GD; NMDA receptor; AMPA receptor; Glutamate; Morris water maze; Sub-lethal; Spatial memory; GluR2; NMDAR1; NMDAR2a; NMDAR2b

1. Introduction

Soman (pinacolyl methylphosphonofluoridate, GD) is a potent organophosphorous compound (OP) that irreversibly binds to acetylcholinesterase (AChE) in both the peripheral and central nervous systems. At lethal doses, accumulation of acetylcholine rapidly leads to seizures, respiratory failure and death. Survivors of acute, sub-lethal doses can experience long-term health and psychological effects (Kawana et al., 2001; McCauley et al., 2001; Ohtani et al., 2004; Kawada et al., 2005). However, past events have shown that most people, especially emergency and medical treatment personnel, exposed during a mass nerve agent incident receive an initial mild or asymptomatic level of exposure (single, multiple or extended) as opposed to an acute symptomatic exposure (Levin and Rodnitzky, 1976; Morita et al., 1995; Gray et al., 1999).

Abbreviations: GD, soman; XGD, dilute soman; MWM, variable start anterograde Morris water maze paradigm; GluR, glutamate receptor; AMPA, alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NMDAR, N-methyl-D-aspartic acid glutamate receptor; NFM, neurofilament medium; NFL, neurofilament light; PIE, post-initial exposure; AChE, acetylcholinesterase.

* Corresponding author. Tel.: +1 410 436 3862; fax: +1 410 436 8377.
E-mail address: erik.a.johnson1@us.army.mil (E.A. Johnson).

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Previous studies have revealed changes in the brain following sub-lethal nerve agent exposure (Burchfiel et al., 1976; Duffy et al., 1979) that involve not only the acetylcholine system, but also the glutamate system (Shih et al., 1990; Lallement et al., 1991, 1992, 1994b; Sparenborg et al., 1992; McDonough and Shih, 1993; de Groot et al., 2001). Excitotoxic injury caused by increased levels of glutamate has repeatedly been shown to cause cognitive dysfunction (Phillips et al., 1998; O'Dell et al., 2000; Faden et al., 2001). Interestingly, studies show changes in the brain following sub-lethal nerve agent exposure that lead to memory and attention deficits that normally involve the hippocampus (Hatta et al., 1996; Nishiwaki et al., 2001; Miyaki et al., 2005).

The role of the hippocampus in complex visuo-spatial learning and memory has been well established. The high concentrations of *N*-methyl-D-aspartate glutamate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptors, which play a key role in hippocampal-mediated learning and memory (Izquierdo and Medina, 1997), also make the hippocampus highly vulnerable to glutamate-induced excitotoxic injury from GD poisoning (Shih et al., 1990; Lallement et al., 1991, 1992, 1994b; Sparenborg et al., 1992; McDonough and Shih, 1993; de Groot et al., 2001). Repeated sub-lethal exposure to OP compounds can raise extracellular glutamate levels in the brain (Singh and Drewes, 1987) and increased glutamate availability can alter glutamate receptor expression (Piehl et al., 1995; Kreutz et al., 1998; Cebers et al., 2001). Additionally, perturbations in NMDA receptor subunit distribution on the neuronal cell surface (Hardingham et al., 2002) or a rearrangement in the ratio of NMDA subunits in individual receptors can change the overall physiology of the receptor and the functionality of the hippocampus (Cebers et al., 1999). Many models of cognitive dysfunction have shown altered glutamate receptor expression (Luthi-Carter et al., 2003; Mishizen-Eberz et al., 2004) and NMDA subunit ratio rearrangement (Mikuni et al., 1998) suggesting that these mechanisms may also contribute to cognitive dysfunction following sub-lethal GD exposure.

Altered cytoskeletal structural protein [e.g., microtubule associated protein 2 (MAP2), neurofilaments (NF), α-II-spectrin] immunoreactivity has been correlated with adverse behavioral effects following CNS injury (Pike et al., 1998; Isaksson et al., 2001; Shaw et al., 2005; Briones et al., 2006; Bruschettini et al., 2006) and distinct losses of MAP2 have been shown in the hippocampus following acute GD exposure (Ballough et al., 1995). Though altered expression of these structural proteins alone does not suffice as evidence for altered inter-neuron communication (Huh et al., 2003), in conjunction with markers of synaptic architecture (e.g., synaptophysin), these markers can be a good indicator of maladaptive plasticity and altered inter-neuron communication (Phillips et al., 1994; D'Ambrosio et al., 1998).

The present study investigated the effects of repeated sub-lethal GD exposure on anterograde visuo-spatial learning and memory and correlated those results to cytoskeletal, synaptic and glutamate receptor protein alterations in the hippocampus. The guinea pig model was used for these studies due to carboxyesterase (CaE) levels and turnover rates for the guinea pig more closely mimic humans than those for either rats or mice (Atchison et al., 2004). Guinea pigs were exposed to multiple, sub-lethal doses of GD ($0.4 \times LD_{50}$) and assessed up to 88 days post-initial injection using the Morris water maze to study long-term learning and memory deficits. The hippocampus of each animal was then probed for altered cytoskeletal, synaptic and glutamate receptor protein expression using Western blot analyses to correlate protein changes with any observed behavioral deficits.

2. Materials and methods

2.1. Animals

Ten-week-old diet restricted male Hartley guinea pigs (Crl:(HA)BR) (Charles River Laboratories, Wilmington, MA) weighing approximately 460 g were used for the behavioral and biochemical sections of this study. The pathophysiology of GD exposure varies depending on weight and fitness (Sipos et al., 2002) and diet control is a simple procedure to detect GD exposure effects in long-term guinea pig studies (Nold et al., 2001). Therefore, these animals were diet controlled rather than free-fed and sedentary as a better correlate to the lifestyles of active military and emergency personnel (Sipos et al., 2002). Animals were fed 60 mg/kg of Harlan Teklad guinea pig diet once daily following GD exposure and MWM trials. Each animal was implanted with an IPTT^{TM}-300 transponder chip (Bio Medic Data Systems, Seaford, DE) for identification purposes and to record body temperature. Temperatures and weights were recorded twice daily just prior to morning GD exposure and afternoon MWM trials. Data were collected post-initial exposure (PIE) and designated as immediate (7–11 days PIE), 1 month (14–28 days PIE), 3 months (84–88 days PIE) and 6 months (154 days PIE). The research environment and protocols for animal experimentation were approved by the institutional animal care and use committee (IACUC) and complies with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). The animal care program at this Institute is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Soman administration

GD (GD-U-2323-CTF-N, purity 98.8 wt%) was obtained and diluted at the US Army Medical Research Institute of Chemical Defense (USAMRICD, Aberdeen Proving Ground, MD). The highest sub-lethal dose of soman (XGD; 11.2 μg/kg or $0.4 \times LD_{50}$) that does not produce acute toxic signs following a single injection (Atchison et al., 2004) was administered subcutaneously in the scruff of neck once a day (MON-FRI) for 10 exposures at 1.0 mL/kg per injection. Signs of GD toxicity were measured twice daily, in the morning within 30 min of exposure and in the afternoon within 30 min of the last MWM trial. Weight and temperature along with either the presence or absence of lacrimation, salivation,

fasciculations and hyperactivity were measured. No animals died as the result of GD exposure.

2.3. Morris water maze (MWM) visuo-spatial complex learning and memory task

The variable start Morris water maze (MWM) anterograde memory task has successfully and reliably measured cognitive impairment in other models of CNS injury (Hamm et al., 1992), including repeated mild excitotoxic brain injury (DeFord et al., 2002). Guinea pigs were trained on the variable start anterograde MWM paradigm with external maze cues (Morris et al., 1982) with modification: a 15 cm platform replaced the 10 cm platform to accommodate the guinea pig (de Groot et al., 2001). Briefly, a light colored fiberglass pool with a 160 cm diameter and a height of 60 cm was located in a 2.4 m × 2.1 m room. The tank was refilled daily with 25 °C water to a height of 50 cm to optimize the observance of visual cues for the guinea pig. Extra-maze cues were positioned around the pool and remained constant throughout the experiment. The guinea pigs were trained to locate the clear plexiglass platform submerged 2 cm below the water surface and further obscured by non-toxic white Tempera paint. For each trial, the guinea pig was placed facing the wall of the pool in one of the four pool quadrants. The pool quadrant was determined randomly for each day and separately for each animal. Each trial began with the guinea pig being placed in the pool and ended when the animal climbed on the platform. The maximum duration allowed was 120 s. For each session, the animal was marked with a large black ink dot on its back for video tracking purposes. Each animal was tracked using a Panasonic BP334 Digital video camera and data were recorded and analyzed using Watermaze™ version 2.6 software (Coulbourn Instruments; Allentown, PA). If the platform was not found after 120 s, the animal was placed directly on the platform for 15 s. At the end of each trial, the animal was toweled dry and placed in a transfer cage with a heating lamp. Assessment of MWM performance consisted of four trials per day (2–5 min inter-trial interval) for 5 consecutive days. Animals were assessed at 3-week-long time points (7–11, 14–18 and 84–88 days PIE). At each week-long time point, the platform was randomly placed in a new quadrant. Trial duration (s), total path distance (cm), and swim speed (cm/s) were recorded for analysis. The number of animals used for each trial was $n = 20$ (days 7–11, 14–18) and $n = 10$ (days 84–88). Data were evaluated by repeated measures ANOVA analysis with significance set at $p \leq 0.05$.

2.4. Western blot analyses

Western blot analyses of hippocampal tissue lysates were conducted as previously described (Johnson et al., 2004). Briefly, guinea pigs were deeply anesthetized using a lethal dose of pentobarbital, hippocampus tissues were rapidly excised, frozen in liquid nitrogen and stored at −80 °C. The tissue was homogenized in ice-cold triple detergent lysis buffer containing a Complete™ protease inhibitor cocktail (Roche Biochemicals; Indianapolis, IN) using a motorized pestle (Caframo; Wairton, ONT) and Tissue Tearer (Biospec; Racine, WI) on ice. Protein concentration was determined using bicinchoninic acid (BCA) micro protein assays (Pierce, Inc.; Rockford, IL). Forty micrograms of protein were loaded per well and separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes and probed with a primary antibody for either NFM (Encor Biotechnology; Alachua, FL), NFL (Encor Biotechnology; Alachua, FL), synaptophysin (Sigma–Aldrich; St. Louis, MO), α-II-spectrin (Biomol International; Plymouth Meeting, PA), NMDAR2a (Sigma–Aldrich; St. Louis, MO), NMDAR2b (Sigma–Aldrich; St. Louis, MO), GluR2/3 (Chemicon; Temecula, CA) or GluR3 (Chemicon; Temecula, CA). Actin (Sigma–Aldrich; St. Louis, MO) was labeled as a loading control. The membranes were then thoroughly rinsed in Tris-buffered saline with Tween 20, incubated with anti-rabbit (Sigma–Aldrich; St. Louis, MO) or anti-mouse (Zymed/Invitrogen; Carlsbad, CA) alkaline phosphatase-conjugated secondary antibody and developed using Enhanced Chemifluorescence reagents (ECF, Amersham; Arlington Heights, IL). The membranes were then imaged using the STORM 860 phosphorimager/fluorimager (Molecular Dynamics; GE Healthcare, Piscataway, NJ). Saline controls, experimental groups (1, 3 and 6 months PIE) and naïve control (unexposed) groups each had an $n = 6$. Transformed data (exposed or saline densitometry value/naïve control densitometry value × 100) were evaluated by ANOVA and a post-hoc Bonferroni-test for selected pairs (i.e., saline v GD for each time point) was applied. Values are expressed as percentage of naive controls and are given as the mean ± S.D. Differences were considered significant at the level of $p \leq 0.05$.

3. Results

3.1. Physiological measurements

Guinea pigs were injected for 10 days (MON-FRI for 2 weeks) with either saline or 0.4 × LD_{50} GD. Changes in body mass from the first to the last exposure (10 day mass change) was significantly different between the sub-lethal GD and saline-treated groups (saline: +32.2 g versus GD: +19.5 g, $p < 0.05$). Total average body temperature readings were also significantly different between the two groups (saline: 101.1 ± 1.6 °C versus GD: 102.0 ± 0.8 °C, $p \leq 0.001$) though tolerance was not observed (XGD day 1 versus XGD day 12, data not shown). Lacrimation was absent in both groups. Excess salivation, fasciculations and hyperactivity were absent in the saline group but appeared sporadically, though not on consecutive measurements, in the GD group. Out of 200 total observations for the GD group, only 5% were marked as hyperactive, 14.5% as fasciculating and 2% as having signs of excess salivation.

3.2. Learning and memory

To determine whether repeated sub-lethal GD administration affected visuo-spatial complex learning and memory, guinea pigs were assessed using the MWM task as described

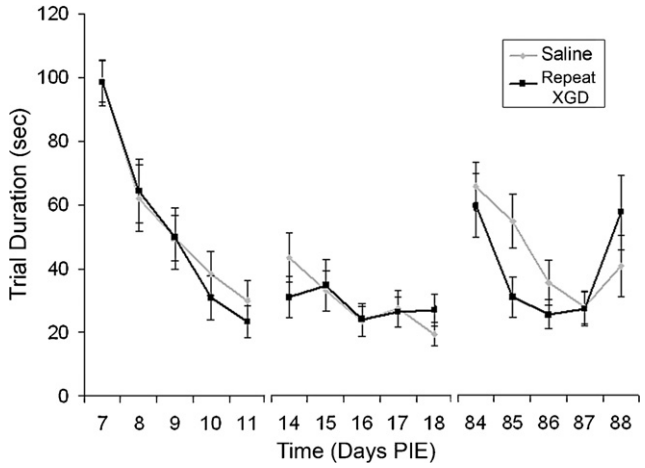

Fig. 1. No learning and memory deficits were detected in GD-exposed guinea pigs using the MWM task. No significant differences were observed between saline-treated and GD-treated group in latency times at any post-initial exposure (PIE) time. Additionally, no differences were observed for distance traveled and average velocity using a one-way ANOVA analysis (not shown). Each data point represents the average trials per day (20 trials per time point per animal,) for each group from an $n = 20$ (days 7–18) or $n = 10$ (days 84–88). Values are reported as mean ± S.D.

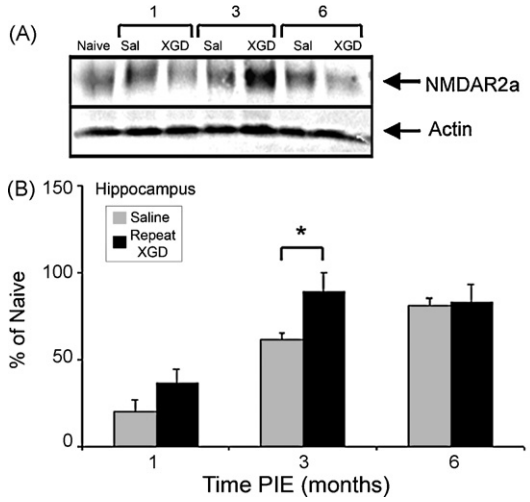

Fig. 2. NMDAR2a densitometry is significantly increased following repeated sub-lethal GD exposure. (A) Representative western blots of NMDAR2a (170 kDa) in hippocampus revealed an increase in NMDAR2a immunoreactivity following repeated sub-lethal GD exposure compared to saline treatment. (B) Densitometric analysis of the NMDAR2a band showed significant increases ($^{*}p < 0.05$) at 3 months post-initial exposure (PIE). Actin (43 kDa) was used as a loading control. Data are given as percent of the naive controls; each time point represents data from an $n = 6$ and is reported as mean ± S.D.

above. Repeated measures ANOVA revealed no significant differences at any MWM time point between the saline group and the GD-treated group in latency (Fig. 1), total path distance or swim speed (data not shown).

3.3. Cytoskeletal and synaptic protein immunoreactivity

To determine whether repeated sub-lethal GD administration led to changes in neurocytoskeletal integrity, densitometric values from Western blots were observed for NFM, NFL, full-length α-II-spectrin and synaptophysin. No significant changes were observed in the hippocampus of the GD-exposed group for any structural protein compared to saline controls at any time point. Similarly, the cytoskeletal protein actin revealed no significant changes in the hippocampus confirming its use as a consistent loading control.

We also observed no significant differences in the immunoreactivity of α-II-spectrin proteolytic breakdown products, indicative of cell death pathway activation (Pike et al., 1998), at 150 kDa (calpain and caspase-3), 145 kDa (calpain-specific) or 120 kDa (caspase-3-specific) at any time point (data not shown).

3.4. Glutamate receptor immunoreactivity

To determine whether repeated sub-lethal GD administration affected NMDA or AMPA glutamate receptor immunoreactivity, Western blot analyses were performed on hippocampal lysates for NMDAR2a, NMDAR2b, NMDAR1, GluR2/3 and GluR3. Densitometric analyses of the NMDAR2a and NMDAR2b subunits revealed significantly greater immunoreactivity of both subunits in the GD-exposed group compared to saline controls in the hippocampus at 3 months PIE ($p \leq 0.05$; for both NMDAR2a and b) and representing an increase from saline controls of 28 and 32%, respectively (Figs. 2 and 3). Analysis of the NMDAR1 subunit revealed a significant decrease in immunoreactivity in the GD-exposed group compared to the saline group by 6 months PIE ($p \leq 0.01$) representing a reduction of 75% from saline controls (Fig. 4).

For AMPA receptors, antibodies for GluR2/3 (which recognizes both GluR2 and GluR3) and GluR3 were used. Densitometric analyses for GluR3 revealed no significant

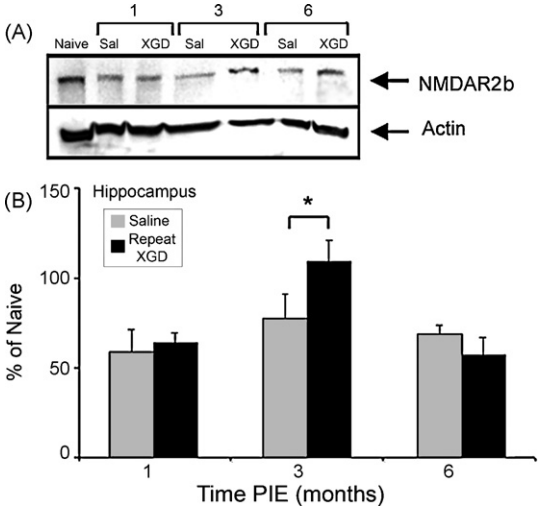

Fig. 3. NMDAR2b densitometry is significantly increased following repeated sub-lethal GD exposure. (A) Representative western blots of NMDAR2b (180 kDa) in hippocampus revealed an increase in NMDAR2b immunoreactivity following repeated sub-lethal GD exposure compared to saline treatment. (B) Densitometric analysis of the NMDAR2b band in hippocampus shows significant increases ($^{*}p < 0.05$) at 3 months post-initial exposure (PIE). Actin (43 kDa) was used as a loading control. Data are given as percent of the naive controls; each time point represents data from an $n = 6$ and is reported as mean ± S.D.

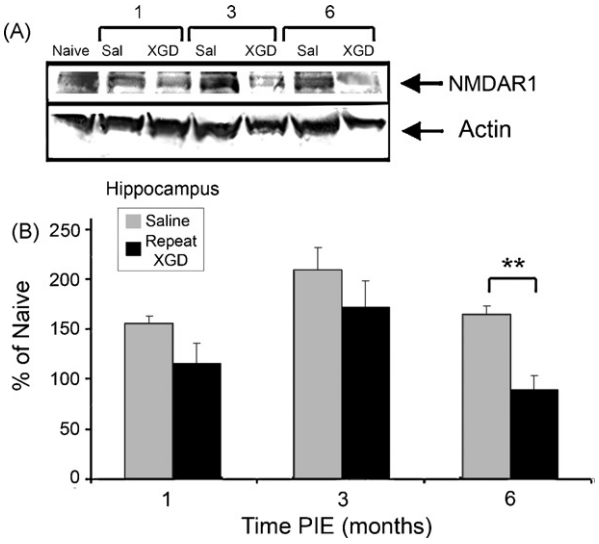

Fig. 4. NMDAR1 densitometry is decreased increased following repeated sub-lethal GD exposure. (A) Representative western blots of NMDAR1 (116 kDa) in hippocampus revealed a decrease in NMDAR1 immunoreactivity following repeated sub-lethal GD exposure compared to saline treatment. (B) Densitometric analysis of the NMDAR1 band showed significant decreases ($^{**}p < 0.01$) at 6 months post-initial exposure (PIE) compared to saline treatment. Actin (43 kDa) was used as a loading control. Data are given as percent of the naive controls; each time point represents data from an $n = 6$ and is reported as mean ± S.D.

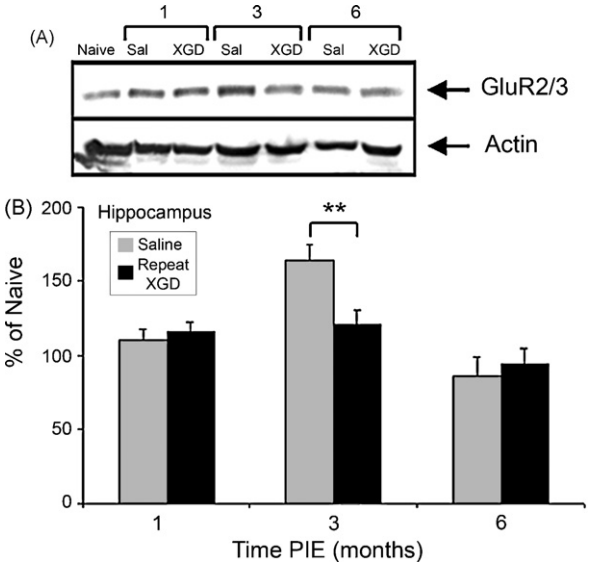

Fig. 5. GluR2 densitometry is significantly decreased following repeated sub-lethal GD exposure. (A) Representative western blots of GluR2/3 (~106 kDa) in hippocampus obtained from repeated sub-lethal GD-treated and saline-treated guinea pigs revealed a decrease in GluR2/3 immunoreactivity following repeated sub-lethal GD exposure compared to saline treatment. (B) Densitometric analysis of the GluR2/3 bands in hippocampus shows significant decreases ($^{**}p < 0.01$) at 3 months post-initial exposure (PIE) thought to be due to decreases in GluR2 alone as analysis of GluR3 alone showed no significant changes (not shown). Actin (43 kDa) was used as a loading control. Data are given as percent of the naive controls; each time point represents data from an $n = 6$ and is reported as mean ± S.D.

difference between the GD-exposed and saline-treated groups (data not shown). However, there was a significant decrease in GluR2/3 immunoreactivity in the hippocampal lysates of the GD-treated group at 3 months PIE ($p \leq 0.01$) representing a decrease from saline controls of 43% (Fig. 5). This, taken with the GluR3 data, suggests a decrease in GluR2 immunoreactivity.

4. Discussion

The present study investigated visuo-spatial memory and cytoskeleton, synaptic membrane and glutamate receptor subunit immunoreactivity in the hippocampus of guinea pigs exposed to repeated sub-lethal doses of GD. This GD-dosing regime revealed significant changes in glutamate receptor immunoreactivity despite no observation of significant changes in visuo-spatial memory or immunoreactivity of structural and synaptic proteins in the hippocampus.

This GD dosing paradigm has been shown to significantly reduce red blood cell (RBC) AChE levels to 9% of controls at the end of 2 weeks of exposure. However, RBC and diaphragm AChE activity significantly recover by 3 days following the last GD exposure (Atchison et al., 2004) which suggests any long-term changes observed are not likely due to continued AChE inhibition. Following repeated, sub-lethal exposures to GD, no statistical difference was observed for latency, swim speed or total path distance between GD-exposed and saline-treated groups in the MWM task despite the successful use of this model by other groups (de Groot et al., 2001; Filliat et al., 2002; Byrnes et al., 2004; Iqbal et al., 2004). These results are consistent with previous work showing no long-term behavioral effects for schedule-controlled behavior (Hymowitz et al., 1990), conditioned avoidance response and passive avoidance tasks following repeated, sub-lethal GD-exposure (Russell et al., 1986). Additionally, MWM performance following repeated sub-lethal exposure to the OP compound, methamidophos, revealed no learning impairments (Temerowski and van der Staay, 2005). However, the dosing paradigm itself may preclude our ability to detect behavioral changes due to OP compound tolerance following repeated administrations (Russell et al., 1986; Hymowitz et al., 1990).

Previous studies of repeated sub-lethal GD exposures have demonstrated pathological and electroencephalographic changes following GD exposure in the absence of behavioral changes (Burchfiel et al., 1976; Duffy et al., 1979; Hymowitz et al., 1990). To determine whether the current GD dosing paradigm led to cytoskeletal and synaptic protein alterations, suggestive of neuronal dysfunction or damage, we investigated long-term protein changes in the hippocampus up to 6 months PIE by examining the expression patterns of three neuron-specific cytoskeletal proteins; NFM, NFL and α-II-spectrin as well as the synaptic vesicle protein synaptophysin. No significant changes in NFL, NFM, full-length α-II-spectrin or synaptophysin immunoreactivity were detected suggesting that the neuronal cyto-architecture remained unaltered. Additionally, no significant changes in the caspase-3 or calpain generated breakdown products of α-II-spectrin were observed indicating that cell death pathways were not activated in agreement with previous work (Churchill et al., 1985; Lallement et al., 1994a; Baille et al., 2001; Carpentier et al.,

2001; Thomson et al., 2005). However, cell death is not necessary for the manifestation of cognitive deficits (Lyeth et al., 1990). Altered immunoreactivity of other proteins, such as glutamate receptors, may also produce cognitive dysfunction.

Both the AMPA- and NMDA-type glutamate receptors play an integral role in learning and memory function as well as OP-induced neural pathology. Specifically, AMPA-type glutamate receptor (GluR) activation is implicated in hippocampal hyperexcitability observed following GD exposure (Sheardown et al., 1990; Lallement et al., 1991; Wood and Tattersall, 2001). Densitometry revealed a significant long-term but transient decrease in GluR2 immunoreactivity in GD exposed animals at 3 months that disappeared by 6 months PIE. The increases in GluR2 levels in the saline control group at 3 months PIE may be reflective of normal developmental changes in glutamate receptor distribution as the animals aged from 10 to 35 weeks, a phenomenon that may be suppressed in XGD exposed animals. Though GluR2 immunoreactivity normally decreases in the hippocampus with age (Gazzaley et al., 1996), larger decreases are seen in Alzheimer's disease (Hof et al., 2002), as with our model, and other neurodegenerative disorders including amyotrophic lateral sclerosis, epilepsy and brain ischemia (Pellegrini-Giampietro et al., 1997; Weiss and Sensi, 2000; Carter et al., 2004; Soundarapandian et al., 2005; Peng et al., 2006; Tortarolo et al., 2006). This suggests that repeated, sub-lethal exposure to GD may render subjects more vulnerable to cognitive dysfunction development especially by exposure to otherwise benign excitotoxic insults (Pellegrini-Giampietro et al., 1997; Munirathinam and Bahr, 2004). These data suggest a transient pathological role for decreasing GluR2 subunit expression in response to repeated sub-lethal GD-exposure though this role is not well defined by this study and requires further investigation.

NMDA-type glutamate receptors, especially in the hippocampus, are integral to learning and memory but are also involved in pathological processes following acute OP poisoning (Churchill et al., 1985; Lallement et al., 1991; McDonough and Shih, 1997). A typical NMDA receptor is a tetramer comprised of a ratio of NMDAR2 subunits, which contribute to the functionality of the receptor, and the main obligatory subunit, NMDAR1 (Wafford et al., 1993; Chazot and Stephenson, 1997; Luo et al., 1997) with each complex having its own distinct pharmacological properties. Significant increases in NMDAR2a and b subunit immunoreactivity occurred in the hippocampus at 3 months PIE but returned to saline-control levels by 6 months PIE. Conversely, NMDAR1 immunoreactivity was comparable between the GD-exposed and saline-treated groups at 3 months but significantly declined by 6 months PIE in the GD-exposed group. This observation may reflect a redistribution of NMDAR2 subunit ratios in response to the increased availability of glutamate in an attempt to maintain the functional homeostasis of the glutamate system at 3 months PIE (Cebers et al., 1999). As an example, increases in NMDAR2a and b can occur in the hippocampus as the result of excitotoxic injury (Sutcu et al., 2005). By 6 months PIE, these subunit ratio alterations may have been functionally insufficient, and in response, the expression of the entire receptor complex (as evidenced by NMDAR1) is likely down-regulated (Cebers et al., 2001).

The ramifications of altered NMDAR subunit distribution following GD exposure are numerous. Increases in NMDAR2a and b subunit expression have been linked to aberrant hippocampal mossy fiber sprouting and epiliptogenesis (Mathern et al., 1996; Mikuni et al., 1998, 1999) and can make NMDAR-expressing neurons particularly vulnerable to excitotoxic insults (Kotapka et al., 1991; Hamm et al., 1993; Back et al., 2004; Mattson et al., 2005). Additionally, decreases in NMDA1 receptors can lead to deficits in areas such as associative memory recall (Nakazawa et al., 2002) and short to long-term memory conversion (Shimizu et al., 2000). Interestingly, spatial memory may be a more resilient cognitive faculty as decreases in hippocampal NMDAR1 expression by as much as 30% do not induce spatial memory deficits measurable by the MWM (Inada et al., 2003). Though this level of decreased immunoreactivity was exceeded at 6 months PIE when compared to saline controls (−75%), it was not if compared to naïve controls (−10%). This may have contributed to our failure to detect behavioral changes, though the successful use of this model by others and the significant reduction of AChE by our dosing regime that produced similar reductions in NMDAR1 indicate otherwise.

In conclusion, the Morris water maze did not detect complex visuo-spatial learning and anterograde memory deficits following repeated sub-lethal $0.4 \times LD_{50}$ GD exposures in guinea pigs. Additionally, Western blot analyses of neuronal cytoskeletal and synaptic membrane proteins revealed no abnormalities in the cyto-architecture or the presence of pathological proteolysis. Results demonstrate that repeated sub-lethal GD-related leads to alteration of both AMPA and NMDA glutamate receptor protein expression within the hippocampus up to 6 months PIE. Though altered expression of glutamate receptor protein has been linked to cognitive dysfunction, the ramifications of these changes following repeated sub-lethal GD exposure require further investigation.

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